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FOREWORD

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Douglas Finner 4/1/98
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Progress Report

Douglas V. Faller, Ph.D., M.D.

Virus-Targeted Therapeutic for Breast Cancer

5. Introduction

This work utilizes our development of small molecules (butyrate-related compounds) to alter the transcriptional response of viral genes for the treatment of human breast cancer. We have previously successfully used these compounds as therapeutic agents for gene regulation in humans.

The transcriptional activity of certain viral genes, including the Epstein-Barr viral Thymidine Kinase gene, is enhanced by these butyrates. This work has led to the development of a virus-directed strategy for treating lymphoid malignancies associated with the Epstein-Barr virus, including Hodgkin's disease, Burkitt's lymphoma, certain non-Hodgkin's lymphomas, and B cell lymphoproliferative disorders. The Epstein-Barr virus, which is associated with these disorders is not normally susceptible to the nucleoside anti-viral agent, ganciclovir, which requires an active viral Thymidine Kinase for its activity. Exposure to Arginine Butyrate, however, induces the latent Thymidine Kinase gene in Epstein-Barr virus-infected cells, resulting in susceptibility to ganciclovir.

We have proposed and successfully carried out (under FDA approval) the treatment of these lymphoid malignancies associated with the Epstein-Barr virus in pilot clinical trials. We are also approved by the FDA to treat EBV(+) solid tumors.

Epstein-Barr virus, in addition to infecting human B cells, can infect epithelial cells. Quite recently, it has been discovered that the Epstein-Barr virus can be found in a wider distribution of lymphoepithelioma-like carcinomas, including the malignant cells of breast cancer. The presence of the Epstein-Barr virus in breast carcinomas of varying histology is strikingly high. EBV is present in 15 of 28 breast cancers. It is most prevalent in medullary carcinomas and is also quite frequent in the (more prevalent) invasive ductal cancers (of 48 invasive ductal carcinomas, 19 were EBV(+)).

In addition to EBV genomic DNA detected by PCR, latent membrane viral protein was detected by immunohistology in the epithelial breast tumor cells (but not in surrounding lymphoid cells). No EBV DNA was found using PCR in 12 normal breast specimens. This finding has raised the possibility of a therapeutic strategy for EBV positive breast cancers. Our approach to developing such a therapy would initially involve investigation of EBV sequences in breast cancer cell lines and specimens, determination of whether treatment with Arginine Butyrate will induce the viral thymidine kinase gene, determination *in vitro* of whether the induction of the TK gene and gene product makes the breast cancer cells now susceptible to ganciclovir, and enrollment patients with breast carcinoma into the FDA-approved EBV-associated malignancy protocol and determination of their responses.

Recent reports demonstrate that Epstein-Barr Virus (EBV)-infection of the malignant cells can be found in as many as 50% of breast cancers. We have recently developed a virus-directed strategy for treating (EBV)-associated malignancies: Hodgkin's Disease, African Burkitt's lymphoma, certain Non-Hodgkin's lymphomas, and B cell lymphoproliferative disorders (EBV-LPD). This strategy is based on induction of the EBV thymidine kinase (TK) gene in latently-infected tumor cells employing the experimental drug Arginine Butyrate. After induction of the viral TK gene by Arginine Butyrate, addition of the FDA-approved nucleoside anti-viral agent ganciclovir (GCV) then leads to specific killing of virus-infected tumor cells which express the viral TK and spares normal cells. This potential therapy does not depend on the associated EBV genome being causally-related to the breast tumor. Just the presence of the EBV genome in latent form would be predicted to make the breast tumor susceptible to this combination protocol. The purpose of this proposal is to explore the association of EBV-infection and breast cancer, and to develop an EBV-based strategy for treating breast cancer.

This Progress Report follows the last (revised) Progress Report by only 6 months, and therefore describes some of the work performed in year 2, which was described in that report also. We have not reproduced data figures, however, presented in that report in this one.

6. Body

Task 1: Refine molecular techniques to determine the presence of the Epstein-Barr virus genome in breast cancer biopsy specimens. This technology is readily available for lymphoid malignancies but needs to be tested for its applicability in epithelial-type tumors like breast cancer. (**ongoing**)

The pathology laboratory techniques in place in this hospital for detection of EBV in breast cancer specimens are twofold. The first is detection of EBERs, and the second is staining for LMP. We have tested two types of epithelial tumors for the presence of EBERs and for LMP, including HIV-associated lymphoepithelioma, nasopharyngeal carcinoma, and breast cancer. Both of these techniques detect the presence of EBV proteins in both the lymphoepithelioma and the nasopharyngeal carcinomas, and both of these tumors are causally-associated with EBV. Therefore, these techniques will detect EBV in epithelial cells. Neither technique has detected EBV proteins in two breast cancer cell lines which we know harbor the EBV genome. This is in agreement with a recent report by others (Glaser, 1998). Whether this is due to lack of expression of these viral genes in breast epithelial cells, or rather are peculiar to the cell lines that we employed in these studies is under investigation. We know that the "gold standard" test for the presence of EBV, in situ DNA analysis, is, by definition, positive in cells harboring EBV, so we can always fall back upon this assay, if necessary, to screen breast tumors for EBV (Chu, 1998). We wish to pursue the question of whether the more widely-accessible pathology laboratory tests for EBV can be employed.

Task 2: Establish dose-response curves for induction of thymidine kinase transcript and protein in Epstein-Barr virus-containing tumors. (**completed**)

Dose-response curves for induction of TK have been performed in 10 tumor cell lines *in vitro*, to date. Concentrations of 0.1 mM butyrate are insufficient for induction in all tumors. The threshold for induction in both epithelial and lymphoid cultures is 0.5 mM. 1-2 mM induced viral TK maximally in 8/10 tumor cell lines. In the two cell lines in which TK induction did not occur at that concentration, no further increase in butyrate concentrations (up to 10 mM) resulted in any induction.

There was no obvious reason for the failure of butyrate to induce TK in the two resistant cell lines, although it was noted that these lines similarly did not growth arrest in response to butyrate. This observation prompted an analysis of the molecular mechanisms underlying cell arrest by butyrate, and the second messengers and coupling of cell cycle and TK gene transcription (discussed below, Task 4).

As proposed, we also studied TK induction in cells with newly-established latent-infections. Two such cells, JY and SPP, were studied. The dose-response for TK induction was identical to that described above. The time course for the induction, however, was quite different (discussed below, Task 3).

The magnitude of TK transcript induction was quite different between the tumor cell lines and the cells with newly-established latent infections. The average TK induction in the EBV-associated tumor cells was 5.6, with a range of 4-11. The average TK induction in the newly-latent cells was 2.3, with a range of 2-4.

TK protein levels follow mRNA levels in dose-response analysis, with, for example, 5-fold induction in RNA paralleling 5-fold induction of protein as assayed by immunoblot.

Task 3: Establish time courses of viral thymidine kinase induction by Arginine Butyrate and its half-life after induction. (completed)

Time courses for induction of TK have been performed in 10 EBV-latent cell lines *in vitro*, to date, with more careful analysis carried out in 5 tumors and 1 newly-latent cell line. 1 or 2 mM butyrate was used to induced viral TK.

In the tumor cells, maximal induction did not occur until 24 hr, with no or minimal induction observed at 6 and 18 hrs. Maximum levels persisted at 48 hrs, the latest time-point studied.

As proposed, we also studied the time-course of TK induction in cells with newly-established latent-infections. The time-course for TK induction was quite different than that was observed for the tumors. In the newly-latent cells, maximal induction occurred at 6 hr (the earliest time point studied, with levels falling at 12 hours and baseline levels at 24 hrs, despite persistent exposure to the drug. Washout studies showed that a 24 hr drug-free interval was necessary for the cells to again become susceptible to TK induction by butyrate.

These same time-courses and differences were found when phorbol ester was used instead

of butyrate as the TK-inducing agent.

Task 4: Analysis of *cis*-acting elements on viral TK promoter responsive to Arginine Butyrate. (in progress)

We believe that the results described under Task 2, above, have provided an insight into the molecular mechanisms regulating viral TK gene transcription by arginine butyrate. The ability of butyrate to induce cell cycle arrest appears to correlate with the ability to induce TK gene transcription. We have therefore continued investigation of the molecular mechanisms of cell cycle arrest induced by butyrate. The results of this study, which was ongoing at the time of the last Progress Report and reported in part, have reached a slightly different conclusion than suggested during that interim report. Our further studies have shown that the mechanisms of cell cycle blockade and TK induction by butyrate are independent of p21 synthesis and p53 synthesis (Vaziri, 1998). This is an important finding, in that it predicts that p53- or p21- deficient tumors should not be refractory to TK induction and killing by the Arginine Butyrate and GCV combination.

Millimolar concentrations of butyrate have been reported to cause a G₁ block, inhibition of cellular proliferation, and inhibition of onset of DNA synthesis in a large number of cultured cell types including Chinese hamster ovary cells, adenovirus-infected and uninfected HeLa cells, 3T3 mouse fibroblasts, normal and SV 40-transformed human keratinocytes, and normal and Rous sarcoma-virus-transformed chicken heart mesenchymal cells. All of these reports are consistent with a butyrate-mediated cell cycle block, thought to be in the G₁ phase of the cell cycle. The butyrate cell cycle block is associated with a putative restriction point related to termination of expression of a labile protein. However, the precise mechanisms of sodium butyrate-induced G₁ arrest have not been elucidated.

Mitogen-stimulated progression through the replicative cell cycle is mediated by intracellular signal transduction events involving generation of second messengers, activation of protein kinase cascades, and gene transcription. It is probable that butyrate-induced growth arrest results from modification or perturbation of these crucial mitogenic signaling events. Indeed, several investigators have demonstrated effects of butyrate on some of the mitogenic signaling events which are considered to be important for normal cell cycle progression.

However, despite reports of correlations between G₁ arrest and perturbation of certain mitogenic signaling events, there exists a certain amount of confusion in the literature regarding the nature of responses of specific signaling events to treatment with butyrate. For example, expression of cyclin D, a key regulator of G₁ progression, has been reported to be both induced and suppressed in response to sodium butyrate. Such discrepancies may result, in part, from the fact that many studies of butyrate action have been performed using tumor-derived cells. Transformed cell lines are inherently genetically unstable, and are likely to have acquired numerous mutations both during tumorigenesis and selection in culture. Potentially, such lesions could have profound effects on mitogenic signal transduction pathways, thereby resulting in idiosyncratic responses to butyrate.

We have attempted to resolve some of the controversy regarding molecular mechanisms of sodium butyrate-induced G_1 arrest. In experiments described here, we have tested the hypothesis that butyrate modifies mitogenic G_1 signaling pathways in the context of a non-transformed cell line with well defined growth properties in culture. We chose to use mouse 3T3 fibroblasts for these studies because these non-transformed cells are stringently growth-regulated and have well-defined growth factor requirements in culture. Moreover, much is known regarding the signal transduction events which mediate responses to positive and negative regulators of growth in these cells. Importantly, many of the findings relating to cell cycle regulation originally documented in 3T3 fibroblasts have since been shown to be applicable to many other cells in culture and *in vivo*. Indeed, results from numerous studies in 3T3 cells have provided a paradigm for mammalian cell cycle control. Cell cycle regulation as exemplified by 3T3 cells will be considered briefly below.

For exponential growth, 3T3 fibroblasts require exogenous growth factors, usually provided by the addition of 10% serum to the culture medium. However, upon transfer to medium containing low concentrations (0.5%) of serum, the cells exit the replicative cycle and undergo growth arrest (a state also termed 'quiescence' or ' G_0 '). Upon re-addition of 10% serum to growth-arrested fibroblasts the cells synchronously re-enter the cell cycle and, after a lag phase of approximately 12 hours (termed ' G_1 '), begin to replicate their DNA. DNA synthesis (S-phase) lasts approximately 6 hours and is followed by another 5-6 hour lag phase (G_2) which precedes mitosis. Following mitosis, cells return to the quiescent state, or continue to cycle, depending upon culture conditions such as cell contact or on the local concentration of growth factors.

Purified fibroblast mitogens, such as Platelet-Derived Growth Factor (PDGF, which accounts for much of the mitogenic activity present in serum), are sufficient to stimulate quiescent cells through G_1 and into S-phase. In order to progress through G_1 into S-phase, cells require continuous stimulation by mitogen up until a point approximately 1 hour prior to S-phase (termed the 'R'-point, probably equivalent to 'START' in yeast). Upon reaching the 'R'-point, the cells become committed to replicate their DNA even in the absence of exogenously added growth factors. Therefore, G_1 is a critical 'decision-making' time in which cells commit to enter S-phase and replicate their genetic material, or remain in a quiescent state. Some of the key molecular signals which influence this decision-making process are described below.

Binding of growth factors (such as PDGF) to appropriate cell surface receptors initiates a series of temporally-ordered intracellular events including activation of small G-proteins, generation of second messenger molecules, and stimulation of protein kinase cascades. These events result in the transcription of genes whose products enable cells to progress through G_1 . Some of the critical signaling events which occur in late G_1 , and are absolutely necessary for initiation of DNA replication, are summarized as follows: Mitogen-induced Cyclin D expression begins during mid G_1 , and results in activation of Cyclin-Dependent Kinases (CDKs) 4 and 6. Active CDK-cyclin D complexes phosphorylate the Rb tumor suppressor protein and also, most probably, other important substrates. Rb phosphorylation results in release of an inhibitory constraint (imposed by hypo-

phosphorylated Rb) upon the E2F transcription factor. Transcriptionally-active heterodimers of E2F and DP (E2F-related proteins) promote the expression of a subset of genes including cyclin E. E2F induction of cyclin E may constitute a positive feedback loop, resulting in further increases in CDK activity, Rb phosphorylation, and 'free' E2F. The products of E2F-regulated genes (including Cyclins E and A) are thought to enable cells to enter S-phase. It is not yet clear precisely how these signals regulate assembly and activation of the DNA replication machinery. Nevertheless, accumulation of sufficiently high levels of E2F is a critical determinant of entry into S-phase. Indeed, forced expression of E2F alone is sufficient to stimulate quiescent cultured cells to replicate their DNA.

Mitogenic G₁ signaling events are also subject to negative regulation. For example, CDK inhibitors (such as p21/CIP/WAF and p27) which are expressed in response to anti-proliferative stimuli (*e.g.* cytokines, genotoxic agents) can inhibit the kinase activity of CDKs, thereby eliciting proliferative arrest.

In experiments described here, we have tested the effects of butyrate on cell cycle progression, and on key signaling events which regulate passage through G₁. We show that butyrate induces expression of the p21 CDK inhibitor at the transcriptional level, and that butyrate-induced cell cycle arrest results in large part from disruption of the Rb signaling axis. Surprisingly however, we have found that p21 is dispensable for butyrate-induced growth arrest. Instead, inhibition of cyclin D1 expression provides a potential mechanism for butyrate-induced arrest in G₁.

Butyrate-induced G1 arrest is p53-independent

The product of the p53 tumor suppressor gene is stabilized and activated in response to a variety of growth-inhibitory stimuli, notably genotoxic agents such as UV light and ionizing radiation. When active, p53 can elicit growth arrest in the G₁ phase of the cell cycle or apoptosis (depending on cell type and nature of stimulus). Since butyrate is known to arrest many cell types in G₁, and is also reported to induce apoptosis in some cell lines, we considered the possibility that p53 may mediate these responses. Therefore, to test this hypothesis we obtained primary cultured Mouse Embryo Fibroblasts (MEFs) which had been derived from wild-type (p53 +/+) and p53-deficient (p53 -/-) transgenic animals. [Cells and Culture Methods: p21 -/- and p53 -/- MEFs and appropriate wild type control cell lines were provided by Drs. P. Leder and T. Jacks respectively.

Murine 3T3 cell lines (Balb 3T3, NIH 3T3, and Swiss 3T3 cells) were obtained from the American Type Culture Collection. Identical results were obtained with all three 3T3 cell lines. All cells were cultured in DMEM containing 10% heat-inactivated bovine serum, supplemented with glutamine and penicillin/streptomycin. HPV E6 and E7-expressing 3T3 cells were generated by retroviral infection as described previously. Stably-infected cells were selected in medium containing 0.5 mg/ml G418. To avoid artifacts due to clonal selection of aberrant cells, experiments were performed with pools of G418-resistant cells. To generate cell lines stably-expressing the Cyclin E promoter-luciferase construct Swiss 3T3 cells were co-transfected with 30 µg of pCyc E -795/+100 and 2 µg of the pCneo construct (Promega) to enable selection of transfected cells. Stably-transfected cells were selected in medium containing 0.5 mg/ml G418. Experiments were performed using pooled colonies of G418-resistant cells.]

To test whether p53-deficiency affected butyrate-induced G₁ arrest, we compared the susceptibility of p53+/+ and p53-/- cells to inhibition of G₁ progression by butyrate. [Mitogenic Assays and FACScan analysis: To elicit growth arrest, near-confluent cultures of cells were placed in medium containing 0.5% serum for 48 hours. Serum-starved cultures were stimulated to enter the cell cycle by addition of fresh serum (to a final concentration of 10%) to the starvation medium. In some experiments 1 μ Ci/ml [³H]-methyl thymidine was added to the cultures at the time of serum stimulation. To determine relative rates of DNA synthesis, the incorporation of tritiated thymidine into genomic DNA was determined by NaOH/SDS solubilization of TCA-fixed cells as previously described. For FACScan analysis, monolayers of cells were washed in PBS, detached from the culture dish with trypsin/EDTA, fixed in 35% ethanol, stained with propidium iodide, then analyzed on a Becton Dickinson flow cytometer as previously described.

We arrested the p53+/+ and -/- MEFs in G₀ by serum deprivation for 48 hrs. The resulting cultures of quiescent cells were stimulated to re-enter the cell cycle with serum in the absence, or presence, of varying concentrations of butyrate. [³H]-thymidine incorporation into genomic DNA was measured to provide an index of entry into S-phase. As shown in **Figure 1**, butyrate prevented entry into S-phase in a dose-dependent manner in both cell lines. The dose-response curves for butyrate-induced G₁ arrest in p53+/+ and p53-/- MEFs were indistinguishable. These data showed that lack of p53 did not preclude G₁ arrest in response to butyrate. Therefore, butyrate-elicited G₁ arrest in a p53-independent manner.

Induction of p21 transcripts during Butyrate-induced G1 Arrest.

Having eliminated a role for p53 in butyrate-induced G₁ arrest, we investigated alternative mechanisms whereby butyrate might inhibit progression through G₁. In recent years, the p21 CDK inhibitor, which negatively regulates cell cycle progression in G₁, has been shown to be expressed at high levels in response to a variety of anti-proliferative stimuli (including some cytokines and DNA damaging agents). We hypothesized that butyrate-induced G₁ arrest may be mediated by changes in p21 expression. p21-mediated G₁ arrest resulting from genotoxic agents is believed to result from a linear sequence of events involving activation of p53, transcriptional induction of the p21 gene by active p53, and inhibition of CDK activities by high level p21 expression. Although our studies with p53-deficient MEFs suggested no role for p53 in butyrate-induced G₁ arrest, it remained possible that butyrate might elicit G₁ arrest in a p21-dependent (yet p53-independent) manner. Indeed, regions of the p21 promoter other than the p53-binding sites have been shown to be responsive to anti-proliferative stimuli (such as those resulting from TGF β or induction of terminal differentiation).

Therefore, we tested the possible involvement of p21 in Butyrate-induced cell cycle arrest. We performed RNA blotting experiments to analyze the expression of p21 mRNA under conditions of butyrate induced cell cycle arrest. [RNA blot analysis: RNA was extracted from cells according to the single step method of guanidine isothiocyanate. 20 μ g samples of total RNA were electrophoresed on formaldehyde/agarose gels as described previously. The separated RNAs were

transferred to nitrocellulose filters and probed with random-primed ^{32}P -labelled cDNAs. Hybridization and high-stringency wash conditions were performed as previously described.] As shown in **Figure 2a**, butyrate-treated 3T3 cells expressed high levels of p21 transcripts relative to control untreated fibroblasts. FACScan analysis of PI-stained nuclei from control and butyrate-treated cells indicated that 92% of the butyrate-treated cells were arrested in G_1 , relative to 54% of control cells (**Figure 2b**). The large fold induction of p21 mRNA levels during butyrate-induced G_1 arrest (**Figure 2**) is comparable to the fold increase of p21 transcription which is elicited by other cytostatic agents.

Potentially, butyrate-induced p21 expression could have resulted from increased transcription of the p21 gene, or increased stability of the p21 transcript. While our studies were in progress, Nakano et. al. (Nakano, 1997.) reported that butyrate induced transcription of heterologous p21 promoter-luciferase constructs in a p53-deficient human colon adenocarcinoma cell line. We have obtained similar results in transient transfection experiments in 3T3 fibroblasts. In our experiments, heterologous p21 promoter-luciferase constructs with deletions or mutations in the p53-binding site retained butyrate-inducibility. Since similar studies have already been published by another group, we have chosen not to duplicate those results here. Nevertheless, our experiments and those of Nakano et. al. have shown that induction of p21 by butyrate can be accounted for by p53-independent transcriptional regulation.

p21 is not required for Butyrate-induced G_1 arrest

The experiments described above indicated a correlation between butyrate-induced p21 expression and G_1 arrest. To test the hypothesis that p21 mediated sodium butyrate-induced G_1 arrest, we studied the effects of butyrate on cell cycle progression in cultures of fibroblasts from p21-deficient (p21 $^{-/-}$) transgenic mice. We stimulated quiescent cultures of p21 $^{+/+}$ and p21 $^{-/-}$ mouse embryo fibroblasts with serum in the presence of varying doses of butyrate. We measured the incorporation of [^3H]-thymidine into genomic DNA to provide an index of G_1 progression and entry into S-phase. Surprisingly, both the p21 $^{+/+}$ and p21 $^{-/-}$ cells were susceptible to butyrate-induced cell cycle arrest in G_1 (**Figure 3**). The dose-response curves for butyrate-induced G_1 arrest in p21 $^{-/-}$ and p21 $^{+/+}$ cells were indistinguishable (**Figure 3**). These data showed that p21 was not required for butyrate-induced cell cycle arrest.

Butyrate inhibition of Cyclin D1 expression

The experiments described above indicated that p21-independent mechanism(s) must account for butyrate-induced inhibition of cell cycle progression. Transitions between different phases of the cell cycle are regulated by the activities of Cyclin-Dependent Kinases (CDKs). CDK activities are in turn dependent upon the expression of appropriate cyclin proteins. Thus progression through G_1 and into S-phase requires the sequential expression of cyclins D, E, and A (and concomitant activation of their CDK partners). Since butyrate arrested cell cycle progression during G_1 , we asked whether mitogen-regulated expression of cyclins was perturbed by butyrate treatment. Therefore, we performed immunoblot analysis of cyclin expression in cell extracts from quiescent, serum-stimulated, and serum plus butyrate-treated 3T3 fibroblasts. As expected, Cyclin D protein

levels were induced (relative to quiescent cells) at time points corresponding to mid-G₁ in serum-stimulated cells (**Figure 4**). However, in the presence of butyrate, serum failed to induce cyclin D expression at these (or any other) time points. RNA blotting experiments showed that butyrate inhibited mitogen-induced accumulation of cyclin D₁ transcripts in 3T3 cells as well as in embryonic fibroblasts from p21^{+/+} and p21^{-/-} mice (**Figure 5**).

To test the specificity of the effect of butyrate on cyclin D₁ expression, we also probed the RNA blot shown in **Figure 5** with a radiolabeled murine cyclin D₂ cDNA. As shown in **Figure 5**, butyrate treatment did not significantly affect cyclin D₂ mRNA levels in any cell line tested. Surprisingly, 3T3 cells and embryonic fibroblasts from p21^{+/+} and p21^{-/-} mice each expressed markedly different basal (and mitogen-stimulated) levels of cyclin D₂ mRNA. The reason for the differential pattern of cyclin D₂ expression between these cell types is not known. Nevertheless, these data showed that cyclin D₁ mRNA expression was inhibited relatively specifically in a p21-independent manner. These results identified inhibition of cyclin D expression as a potential mechanism for p21-independent butyrate-induced growth arrest. Indeed, butyrate inhibited cyclin D₁ expression and G₁ progression with a similar dose-dependencies (please compare butyrate concentrations used in **Figures 3** and **5b**, for inhibition of mitogenesis and cyclin D expression in p21^{-/-} cells respectively).

Mechanism of inhibition of Cyclin D1 expression by butyrate.

The butyrate-induced changes in cyclin D₁ expression could potentially have resulted from decreased stability of cyclin D₁ mRNA or from reduced transcription of the cyclin D₁ gene. To distinguish between these possibilities, we performed transient transfection experiments using a heterologous reporter gene construct containing the promoter region of the cyclin D₁ gene linked to a luciferase reporter cDNA (designated -1745 D₁-LUC). Cultures of 3T3 cells were co-transfected with the cyclin D₁ promoter-luciferase construct and a PDGFR promoter-Chloramphenicol Acetyl-Transferase (CAT) reporter construct (as an internal control). The transfected cultures of fibroblasts were rendered quiescent by serum-starvation for 24 hours. The resulting cells were then incubated for an additional 12 hours with 10% serum in the absence or presence of 5 mM butyrate (or with no serum as a control). At this time, cytosolic extracts were harvested from the cells as described in 'Materials and Methods'. Each extract was assayed for both luciferase and CAT activity (to provide an indication of cyclin D₁ and PDGFR promoter-driven reporter gene expression respectively). As expected, serum stimulation induced expression of the cyclin D₁ promoter-driven luciferase reporter gene relative to control quiescent cultures. However, in the presence of butyrate, mitogen-stimulated luciferase activity was fully attenuated (**Figure 6**). Therefore, butyrate prevented serum-dependent activation of the cyclin D₁ promoter. The effect of butyrate on the cyclin D₁ promoter was relatively specific, since PDGFR promoter-dependent transcription of a CAT cDNA in the same cultures of 3T3 cells was unaffected by butyrate (**Figure 6**). These data demonstrated that relatively specific transcriptional mechanisms could account for inhibition of cyclin D₁ expression in response to butyrate.

Butyrate perturbs G1 signaling events distal to cyclin D expression

When activated, cyclin D-dependent kinases (CDKs 4 and 6) phosphorylate multiple sites

upon the Retinoblastoma (Rb) tumor suppressor protein. Phosphorylation of Rb by CDKs relieves an inhibitory constraint which is imposed by hypo-phosphorylated Rb upon the E2F transcription factor family of proteins. Thus, phosphorylation of Rb by CDKs enables the transcriptional activation of E2F-regulated genes (including those encoding Cyclins E and A, DHFR, and many others) whose products are thought to mediate entry into S-phase. As described above, butyrate treatment prevented mitogen-induced expression of cyclin D₁. Therefore, we tested whether inhibition of cyclin D by butyrate was sufficient to perturb more distal mitogenic events, namely Rb phosphorylation and activation of E2F-dependent promoters.

Hyper-phosphorylated Rb protein migrates with a characteristic retarded electrophoretic mobility (relative to hypo-phosphorylated Rb) on SDS-polyacrylamide gels. We tested whether butyrate treatment affected the phosphorylation of Rb, a process known to be carried out by active cyclin D-CDK complexes during mid-late G₁. We performed immunoblot analysis of cytosolic lysates from quiescent, serum-stimulated, and serum plus butyrate-treated cells using an anti-Rb antibody. As expected, serum-stimulation elicited a band shift characteristic of hyperphosphorylated Rb in both p21^{+/+} and p21^{-/-} mouse embryo fibroblasts (**Figure 7a**). However, this mobility shift did not occur in Rb present in lysates from butyrate-treated cells (**Figure 7a**). Therefore, butyrate prevented mitogen-dependent phosphorylation of Rb in a p21-independent manner.

As already noted, hypo-phosphorylated Rb negatively regulates E2F activation and entry into S-phase. Entry into S-phase is mediated by transcriptional induction of E2F-regulated genes, including cyclin E. We tested whether butyrate inhibition of Rb phosphorylation was sufficient to perturb downstream E2F-dependent events. We generated a 3T3 cell line stably-expressing a plasmid containing 895 bp of the 5' region of the murine cyclin E gene linked to a luciferase reporter cDNA. Mitogen-regulated transcription of the luciferase reporter gene from this construct is entirely dependent upon E2F sites within this region of the promoter. As expected, serum induced an increase (approximately 8 fold-in the experiment shown) in the expression of cyclin E promoter-driven luciferase activity in the stably transfected cells (**Figure 7b**). Serum-induced luciferase activity was inhibited by sodium butyrate in a dose-dependent manner (**Figure 7b**). The dose-response curves for inhibition of cyclin E promoter activity by butyrate were similar to the values we obtained for inhibition of mitogenesis and cyclin D expression.

To further test our hypothesis that Rb-dependent events are perturbed by butyrate, we performed immunoblot analysis of cyclin E and cyclin A proteins (whose mitogen-induced expression is mediated by E2F sites within the promoter regions of the genes encoding these proteins) in quiescent, mitogen-stimulated, and mitogen+butyrate-treated cells. As shown in **Figure 7c**, the serum-dependent expression of both cyclins A and E was prevented by butyrate, concomitantly with inhibition of Rb phosphorylation. Overall, the data described above showed a good correlation between perturbation of the Rb signaling pathway and butyrate-induced G₁ arrest.

HPV-E7-expressing cells display increased resistance to Butyrate-induced G₁ arrest

Our data suggested that butyrate-induced disruption of the Rb signaling pathway may cause G₁ arrest. To test this hypothesis we adopted a 'loss of function approach' and engineered 3T3-derived cell lines with defects in Rb function. When expressed, certain viral oncoproteins such as the Human Papilloma Virus (HPV) E7 protein, bind to and sequester Rb and the Rb-related p107 and p130 pocket proteins. The binding of Rb by viral oncoproteins abrogates the requirement for Rb phosphorylation which ordinarily regulates E2F transcriptional activity. De-regulated E2F activity in HPV E7-expressing cells is thought to contribute to defective checkpoint controls and aberrant proliferation. We have used retroviral vectors to generate 3T3 cells expressing stably-integrated HPV E7 genes. As expected, the resulting E7-expressing cells displayed high levels of E2F activity, grew to high density, and showed a high degree of serum-independent proliferation relative to control cell lines which were infected with an 'empty' retroviral vector (data not shown). Therefore, E7 expression resulted in loss of normal Rb-mediated checkpoints in these cells.

We compared the butyrate-sensitivity of mitogen-stimulated G₁ progression in control cells expressing vector alone (designated 'SXS_N'), cells expressing the HPV E6 gene (which when expressed elicits ubiquitination and proteolysis of p53), and cells expressing stably-integrated HPV E7 alone or in combination with the E6 gene (designated 'SE7' and 'SE6E7' respectively). As expected, abrogation of p53 function by E6 expression had no effect on sensitivity to butyrate-induced G₁ arrest. However, cell lines expressing HPV E7 (alone or in combination with E6) displayed markedly increased resistance to butyrate-induced growth arrest. As shown in **Figure 8**, a dose of 3 mM butyrate, which elicited 80% inhibition of S-phase entry in control (or HPV E6-expressing) cells, only inhibited entry into S-phase by approximately 38% in HPV E7-expressing fibroblasts. These data showed that functional inactivation of Rb (and other pocket proteins), but not of p53, resulted in decreased sensitivity to the cytostatic actions of butyrate. These data support our hypothesis that butyrate-induced G₁ arrest in normal cells results in large part from a p53/p21-independent perturbation of the Rb signaling axis.

Interpretation of Findings

Although butyrate is known to inhibit cell cycle progression, the molecular basis of butyrate-induced G₁ arrest has not been elucidated. We and others have hypothesized that inhibition of cell cycle progression in response to butyrate results from modification of mitogenic signal transduction event(s) during G₁. In this report, we have tested the effects of butyrate on mitogen-induced G₁ signaling events. Since p53 mediates growth inhibition in response to a variety of stimuli, we initially investigated a possible role for p53 in butyrate-induced G₁ arrest. Our results demonstrate that p53-deficient cells (p53^{-/-} MEFs from transgenic animals, and fibroblasts expressing the HPV E6 oncoprotein in which p53 levels and activity are markedly reduced) remain susceptible to butyrate-induced growth arrest. These data suggest that p53 does not mediate butyrate-induced G₁ arrest. Since approximately 50% of human tumors have defects in p53 signaling, our finding that p53-compromised cells are responsive to butyrate-induced growth inhibition further emphasizes the potential value of butyrate and related compounds for cancer therapy.

Interestingly, we have shown that expression of the p21 CDK inhibitor gene (which is a mediator of p53-dependent G₁ arrest) is induced by butyrate in a p53-independent manner in untransformed 3T3 cells (as well as in transformed 3T3 lines). These data corroborate recent results from other workers who have also demonstrated p53-independent transcriptional induction of p21 in colon cancer cells. Since p21 inhibits the activities of CDKs which regulate cell cycle progression, these data suggested a potential mechanism for butyrate-induced G₁ arrest. Therefore we tested the hypothesis that p21 may mediate growth arrest in response to butyrate. We examined the susceptibility of p21-deficient cells to butyrate-induced G₁ arrest. Surprisingly, p21^{-/-} cells (from transgenic animals with a p21-null genetic background) were as sensitive as p21-proficient (p21^{+/+}) cells to butyrate-induced growth arrest. These unexpected results suggested the existence of p21-independent pathways of butyrate-induced growth arrest.

In order to identify putative p21-independent butyrate-induced lesions in G₁ mitogenic signaling events we analyzed the expression of the G₁ cyclins in cultures of synchronized fibroblasts. Cyclins are known to regulate transitions between different phases of the cell cycle. Cyclin D, which is transcriptionally induced during mid-G₁, is the first cyclin to be expressed following mitogen-stimulated entry into the cell cycle. Our experiments have shown that butyrate inhibited mitogen-dependent expression of cyclin D₁ mRNA and protein. The dose-dependency for inhibition of cyclin D expression by butyrate paralleled that of G₁ arrest in p21-deficient (and p21-proficient) cells. Therefore, inhibition of cyclin D expression provides a mechanism for p21-independent butyrate-induced G₁ arrest. Interestingly, it has been suggested previously that the butyrate cell cycle block is related to termination of expression of a labile protein. Potentially, Cyclin D may represent this (putative) labile protein.

Consistent with a putative role for cyclin D suppression in butyrate-induced G₁ arrest, we have shown that mitogenic events temporally distal to cyclin D expression (CDK activation and Rb phosphorylation, Cyclin E expression, Cyclin A expression) are inhibited by butyrate. Furthermore,

abrogation of a requirement for cyclin D-dependent kinase activity and Rb phosphorylation by expression of the HPV E7 oncoprotein conferred resistance to the growth-inhibitory actions of butyrate. Overall, these data suggest that butyrate elicits G₁ arrest by perturbing the mitogen-dependent induction of Cyclin D₁ (and consequently also Rb-mediated signaling events which normally occur distal to cyclin D expression). Interestingly, Wintersberger et al. noted that SV 40-transformed cells displayed reduced sensitivity to butyrate-induced G₁ arrest. Like the HPV E7 oncoprotein, SV 40 large T-antigen contributes to cellular transformation by binding and inactivating Rb (and other pocket proteins). Therefore, abrogation of a requirement for cyclin D-dependent kinase activity in SV 40-transformed cells is likely to have conferred the increased resistance to butyrate which was observed in the experiments of Wintersberger and colleagues.

Although HPV E7 expression in our study (and SV 40 transformation in the report by Wintersberger et. al.) did confer significant resistance to butyrate-induced G₁ arrest, high concentrations of butyrate did eventually inhibit entry into S-phase in these experiments. Cyclin E and A-Dependent Kinases are normally activated subsequent to Cyclin D-CDK, and are essential for progression into S-phase, even in the absence of functional Rb. It is possible that induction of p21 expression by butyrate, and p21-mediated inhibition of Cyclin E and Cyclin A-dependent kinases elicited G₁ arrest in the viral oncoprotein-expressing cells. Therefore, our data suggest the existence of at least two butyrate-induced checkpoints during G₁: one at the level of Cyclin D₁ expression, another resulting from p21 induction.

At least two other groups have noted effects of butyrate on the expression of cyclin D₁. Siavoshian et al. demonstrated an increase in cyclin D expression in butyrate-treated HT 29 (human colon cancer) and HBL-100 (mammary epithelial) cells. In contrast, Lallemand et. al. showed that butyrate inhibited cyclin D expression in Benzo[a]pyrene-transformed BP-A31 fibroblasts. Our data are similar to those reported by the latter group. The murine fibroblast cell lines which we have used in our experiments are likely to be more closely related to the BP-A31 cell line (a fibroblast line originally derived from Balb/c-3T3 fibroblasts) used by Lallemand et al. It is possible that mesenchymal cells (such as the ones used in our study and the experiments of Lallemand and colleagues) respond differently from epithelial cells (*e.g.*, those used by Siavoshian and co-workers) with respect to cyclin D expression. Interestingly, butyrate was found to stimulate p21 expression in HT-29 cells. Therefore, it is likely that induction of p21 (but not changes in cyclin D₁ expression) resulting from butyrate treatment contribute to G₁ arrest in this cell type.

Experiments are currently underway in our laboratory to understand mechanisms whereby butyrate perturbs cyclin D₁ expression in normal untransformed mesenchymal lines. Lallemand et. al. performed transient transfection experiments in which they tested the effect of butyrate on reporter gene expression from heterologous constructs containing the human cyclin D promoter. In these studies, which were performed in transformed murine fibroblasts, luciferase transcription directed by the human cyclin D₁ promoter was inhibited by butyrate. Although the 5' region of the murine cyclin D₁ gene has not yet been described, it is likely that the murine cyclin D₁ promoter is similarly affected by butyrate. Transcription of the cyclin D₁ gene is thought to require the prior

transduction of mitogen-regulated signaling events (including second messenger signals, protein kinase cascades, and transcription of immediate-early response genes). The specific signaling cascades and transcription factors which mediate mitogen-dependent expression of the cyclin D₁ gene have not yet been identified. However, the promoter region of the (human) cyclin D₁ gene is known to be responsive to ectopically-expressed *v-ras* and *c-jun* proto-oncogenes. Both *c-ras* and *c-jun* proteins are considered to play important roles in mitogen-stimulated cell cycle progression, and may have physiologically relevant roles in regulation of cyclin D₁ expression. Therefore, signaling cascades involving *ras* and *c-jun* represent potential targets for the growth-inhibitory actions of sodium butyrate. Potentially, butyrate may perturb the ordered regulation of these mitogen-induced second messenger and transcriptional events. Alternatively, butyrate might exert a direct effect on transcriptional regulation of the cyclin D₁ gene, for example direct modification of transcription factor/DNA interactions at the cyclin D₁ promoter.

Many studies have shown that butyrate and related compounds affect chromatin structure and gene expression. Butyrate is known to inhibit the activity of histone de-acetylases. Recent studies suggest that histone acetylation is an important transcriptional regulatory mechanism. Histone acetylation can alter nucleosomal positioning resulting in de-repression of gene transcription. It is possible therefore that many of the effects of butyrate on gene expression result from changes in the acetylation state of histones. Cell cycle progression requires regulation and integration of numerous transcriptional events. It is possible that de-regulation of these events by butyrate (due to changes in histone acetylation) perturbs progression through G₁. Yoshida and Beppu have reported that Trichostatin A, a potent and selective inhibitor of histone deacetylase activity, can elicit arrest in G₁ and in G₂ in Rat 3Y1 cells. We have obtained similar results in experiments with 3T3 cells. It is possible, therefore, that butyrate-induced G₁ arrest results, at least in large part, from hyper-acetylation of histones. Our laboratory has recently developed a series of butyrate analogues and derivatives for treatment of β -thalassemia and other disorders of globin expression. Not all of these compounds affect histone deacetylase activity. We plan to test whether these compounds affect cell cycle progression and mitogen-regulated signaling events (*e.g.*, cyclin D₁ expression). These experiments may enable us to establish correlations (or dissociate between) inhibition of histone deacetylase and cell cycle regulated events (*e.g.*, p21, Cyclin D₁ expression and G₁ progression).

In summary, we have shown that butyrate elicits G₁ arrest in large part by perturbing the Rb signaling axis. At least two distinct mechanisms contribute to butyrate-induced G₁ arrest: (i) inhibition of cyclin D₁ expression and (ii) p53-independent induction of p21. Further studies are in progress to identify the specific molecular lesions which mediate changes in cyclin D₁ and p21 expression.

Task 5: Enroll patients with Epstein-Barr virus-associated malignancies into the FDA-approved Phase I clinical trial. (ongoing)

See **Table I** for current patient accrual and results.

Task 6: Ongoing biochemical and molecular analysis of specimens obtained from patients with Epstein-Barr virus-associated malignancies, to determine the sensitivity of these tumors to Arginine Butyrate alone, to ganciclovir alone, and to the combination of drugs, and to correlate patient responses with *in vitro* sensitivity.

As stated in the proposal, and required by the DOD Human Subjects approval, this Task is dependent on the unpredictable ability to obtain fresh tumor specimens suitable for culture prior to the initiation of the protocol, without putting the patient through additional surgical procedures. Although we have obtained pathology specimens of tumor tissue prior to treatment, and even obtained pathology specimens after treatment in 3 patients, we have only obtained one tumor specimen which produced a tumor line in culture. The data described below on this line now published (Mentzer, 1998), and will not be displayed herein unless requested.

The patient's peripheral blood mononuclear cells were grown in Dulbecco's Modified Eagle's Medium, ME) with 2,000 mg/L glucose (Sigma, St. Louis, MO), supplemented with 10% heat inactivated fetal calf serum (Sigma), 10 mM Hepes buffer, and 2 mM L-glutamine. With spontaneous growth of the cell line, the cells were passaged twice per week for more than eight months. The established cell line expressed a B lymphocyte phenotype identical to the primary tumor (κ^+ , CD19⁺, CD20⁺, CD21⁺, CD23⁺, CD30⁺, CD40⁺, and CD10⁻). Southern blot analysis of the cell line demonstrated integrated genomic EBV DNA. These similarities between the established cell line and the primary tumor permitted use of the cell line in studies of arginine butyrate-induced TK expression.

The cell line was cultured with various concentrations of arginine butyrate, harvested at various time points, and studied for the induction of the TK transcript by RNA blot analysis. These studies demonstrated undetectable basal levels of the TK transcript, consistent with the latent state of viral replication of the EBV-associated cell line. In contrast, treatment of the cell line with arginine butyrate resulted in a dramatic induction of TK transcription within 12 hours.

Treatment of the established cell line with arginine butyrate alone and at concentrations ranging from 1 μ M to 500 μ M did not inhibit cell line proliferation. Similarly, treatment of the cell line with ganciclovir alone at concentrations up to 30 μ M did not inhibit proliferation. In contrast, the combination of arginine butyrate at 500 μ M plus ganciclovir at 10 and 30 μ M synergistically inhibited cell proliferation measured by increases in fluorescence [RFU = relative fluorescent units] of the cytoplasmic label calcein. Assays of cell viability demonstrated that ganciclovir at 10 μ M, 30 μ M and 100 μ M showed synergy with arginine butyrate at 500 μ M or 1000 μ M in decreasing cell viability at days 3 and 6 as defined by nuclear labeling with membrane impermeant fluorescent dye. No differences between treatment groups were observed at day 1 (not shown). These effects on tumor cell proliferation and viability were observed at concentrations of arginine butyrate and ganciclovir anticipated to be achievable *in vivo*. This patient also had a complete response to the combination of Arginine Butyrate and GCV.

Task 7: Ongoing pharmacokinetic analysis of Arginine Butyrate in patients in the Phase I trials. **(ongoing)**

We are continuing to collect samples on treated patients and are analyzing formal pharmacokinetics on Arginine Butyrate in these patients treated with the combination protocol, and have registered this data with the FDA in conjunction with our IND.

Task 8: Use *in vitro* data to modify and re-assess the Phase I clinical trial protocol. **(ongoing)**

None of the *in vitro* data to date suggests that we need to substantially modify our Phase I/II protocol. The profile of adverse events is favorable. The *in vitro* data on TK induction and time-course suggest that short courses of Arginine Butyrate may be insufficient for optimal induction, and our current approach of sustained infusion may be optimal, or, at least, necessary.

Task 9: Prepare and submit FDA report for Phase I trial completion. **(not initiated)**

Task 10: Prepare and submit the Phase II protocol for treatment of Epstein-Barr virus-positive breast malignancies to the FDA. Preparation includes a meeting with the FDA regarding the study and submission of IND. **(not initiated)**

Task 11: Analysis of other agents, including the oral agent Isobutyramide, for their effects on induction of thymidine kinase in EBV-positive tumor cells and sensitization to ganciclovir treatment. **(ongoing)**

Isobutyramide is being studied in parallel with Arginine Butyrate in the extended induction and time course studies (**Tasks 2 and 3**), and also in cytotoxicity testing with GCV in the tumor cell lines (**Task 4**). Isobutyramide appears as efficient as Arginine Butyrate in induction of TK transcript. TK protein production has not yet been analyzed. Early cytotoxicity assays demonstrate synergistic killing with GCV.

Task 12: Formal preclinical toxicity testing of Isobutyramide, in preparation for IND submission. **(ongoing)**

Two species 28-day toxicology (in beagle dog and rat) is complete. Metabolism and distribution studies are complete. Mutagenicity testing is complete. Results will be registered with the FDA. Stability studies not yet initiated.

Task 13: (months 34-36):

Submission of IND for the combination of Isobutyramide and ganciclovir in the treatment of Epstein-Barr virus associated breast cancer. **(not initiated)**

7. Conclusions

In the first 24 months of research under this grant, we have completed several of the Tasks we had set for months 1-24 (Tasks 1, 2, and 3, although we will continue to study mechanisms of resistance of TK induction). The new data we have generated concerning molecular mechanisms of butyrate action on gene expression is contributing to Task 4. We have enrolled several new patients onto the clinical protocol, with pharmacokinetic studies collected, safety assessed, and responses noted (Tasks 4-7).

The development of new, orally-bioavailable forms of TK-gene regulating agents is underway (Tasks 11 and 12).

We will continue work as originally outlined and planned in the SOW.

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8. Appendices

Figures: 1-10

Table: 1

Figure 1. Dose response curves for inhibition of mitogenesis by butyrate in p53+/+ and p53-/- MEFs

Quiescent cultures of p53+/+ (○) or p53-/- (●) MEFs were stimulated with 10% serum for 24 hours without or with varying doses of sodium butyrate and 1 μ Ci/ml of [3 H]-thymidine. After 24 hours of serum stimulation, the incorporation of tritiated thymidine into genomic DNA was determined by scintillation counting of solubilized nuclei as described in 'Materials and Methods'. Data points are means of duplicate determinations which differed by less than 5%. Error bars have been omitted for clarity.

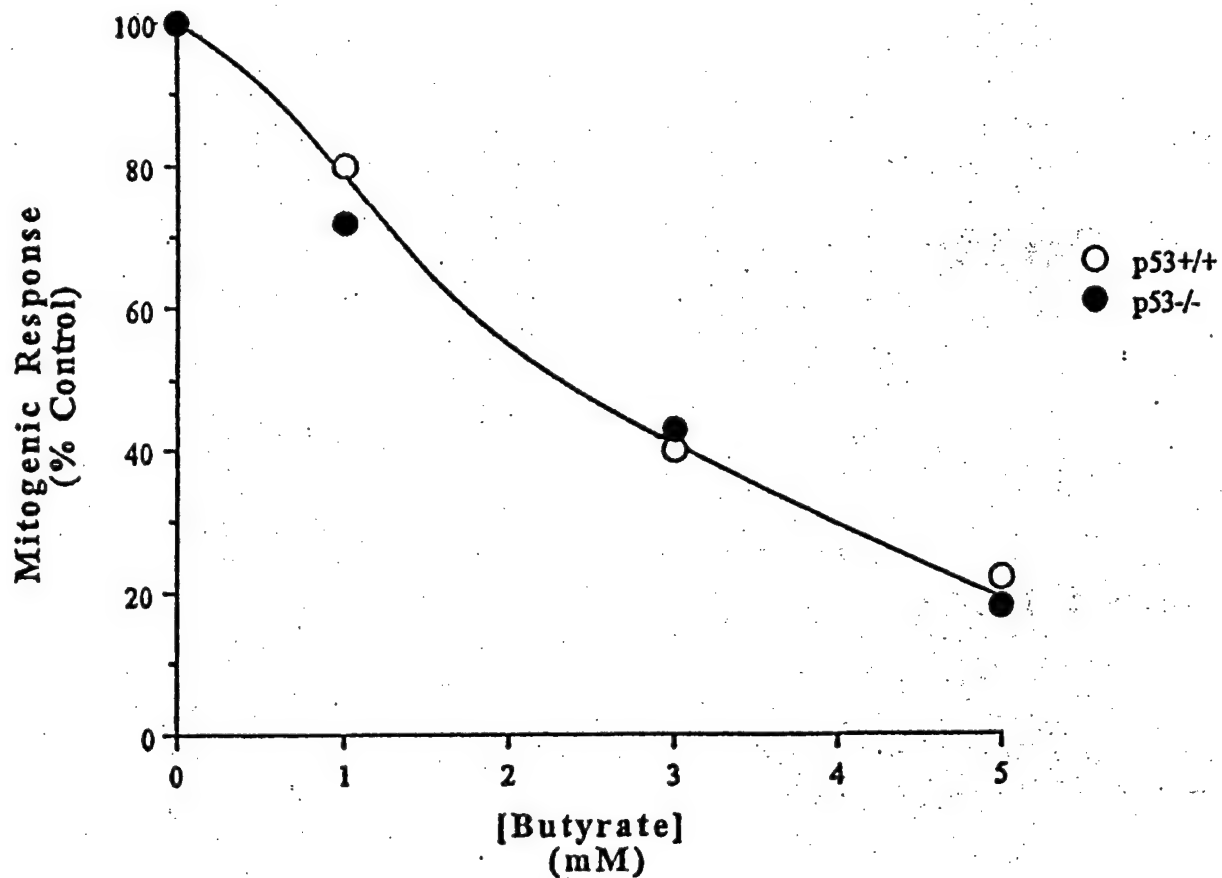


Figure 2. Induction of p21 transcripts and G₁ arrest by butyrate in 3T3 fibroblasts

Exponentially-growing cultures of 3T3 fibroblasts were treated with the indicated concentrations of butyrate for 24 hours. RNA samples from control and butyrate-treated cells were analyzed for p21 expression by RNA blot analysis (a). In a parallel experiment growing cells were placed in fresh serum-containing medium with or without 5mM butyrate, or in medium containing reduced-serum (0.5%).

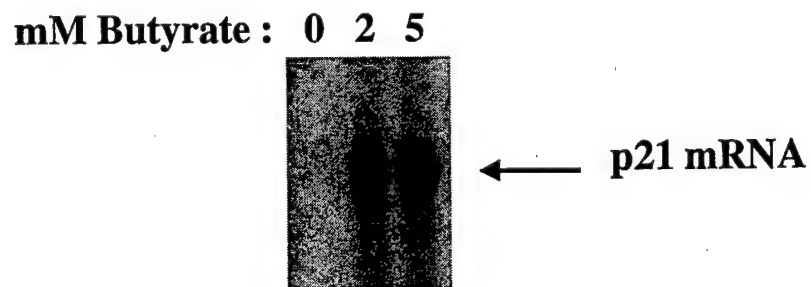


Figure 2. Induction of p21 transcripts and G₁ arrest by butyrate in 3T3 fibroblasts

(b) 24 hours later, the fibroblasts were analyzed for cell cycle distribution by FACScan analysis.

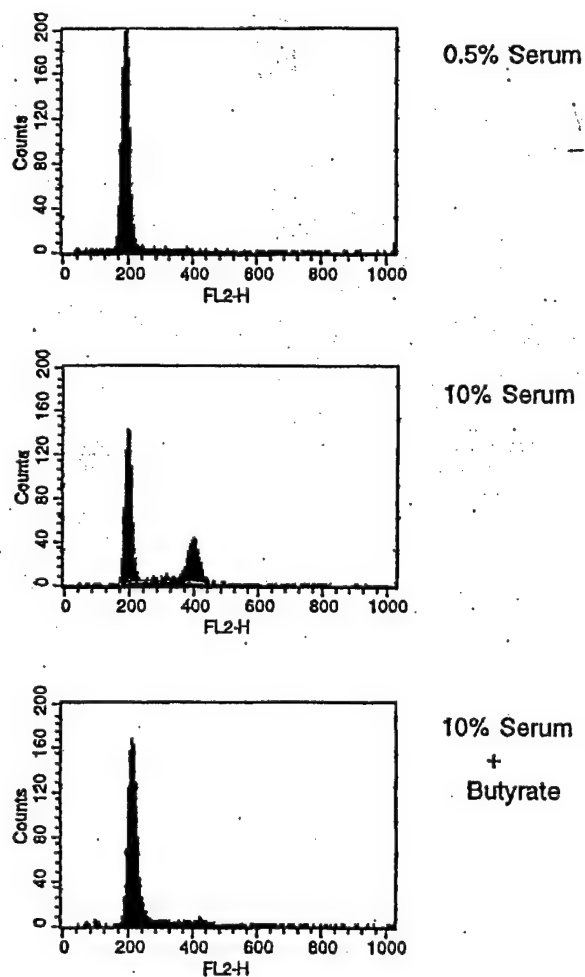


Figure 3. Dose-response curves for inhibition of mitogenesis by butyrate in p21+/+ and p21-/- MEFs

Quiescent cultures of p21+/+ (●) or p21-/- (○) MEFs were stimulated with 10% serum for 24 hours without or with varying concentrations of sodium butyrate and 1 μ Ci/ml of [3 H]-thymidine. 24 hours later the incorporation of [3 H]-thymidine into genomic DNA was measured. Data points are means of duplicate determinations which differed by less than 5%. Error bars have been omitted for clarity.

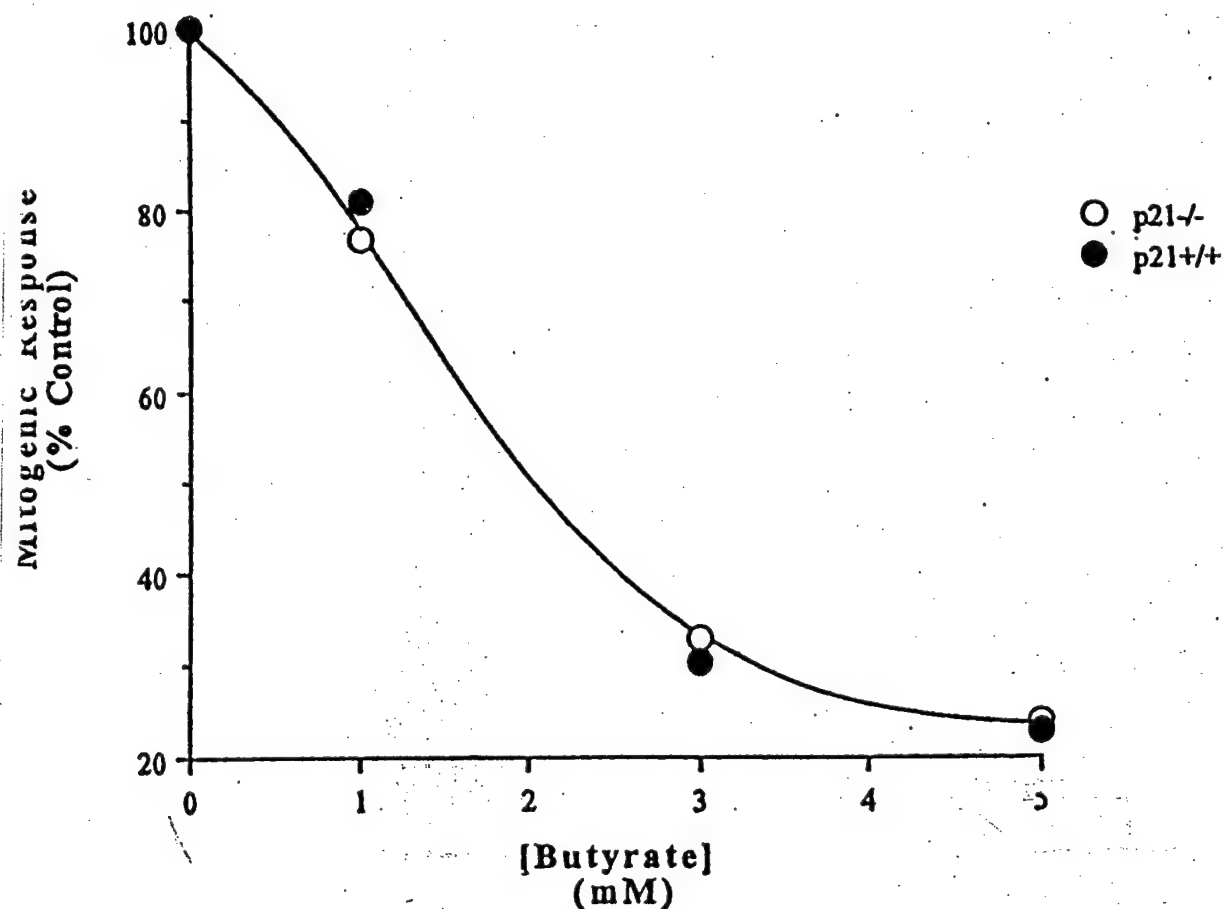


Figure 4. Effects of butyrate on mitogen-induced cyclin D protein in 3T3 cells

Quiescent cultures of 3T3 cells were stimulated with 10% serum without, or with 5 mM sodium butyrate for 12 hours. At this time cytosolic extracts were prepared from the cells. 100 µg of each cytosolic extract was separated on a 10% polyacrylamide gel. After transfer to nitrocellulose, samples were probed with polyclonal antisera to cyclin D. Bound antibodies were visualized using alkaline phosphatase-conjugated secondary antisera.



Figure 5. Effect of butyrate on mitogen-induced cyclin D expression in p21+/+ and p21-/- MEFs

(a) Quiescent cultures of 3T3 fibroblasts, p21+/+ MEFs, and p21-/- MEFs were stimulated with 10% serum without, or with 5mM sodium butyrate. 8 hours later RNA was extracted from the cells. 20 µg of total RNA from each sample was electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and hybridized with a random-primed cyclin D₁ cDNA probe. The upper panel of the figure shows an autoradiogram of the washed blot. The middle panel shows an autoradiogram of the same blot after reprobing with a random-primed cyclin D₂ cDNA. The lower panel is a photograph of the ethidium bromide-stained 28S and 18S RNAs and provides a control for equivalent loading of samples.

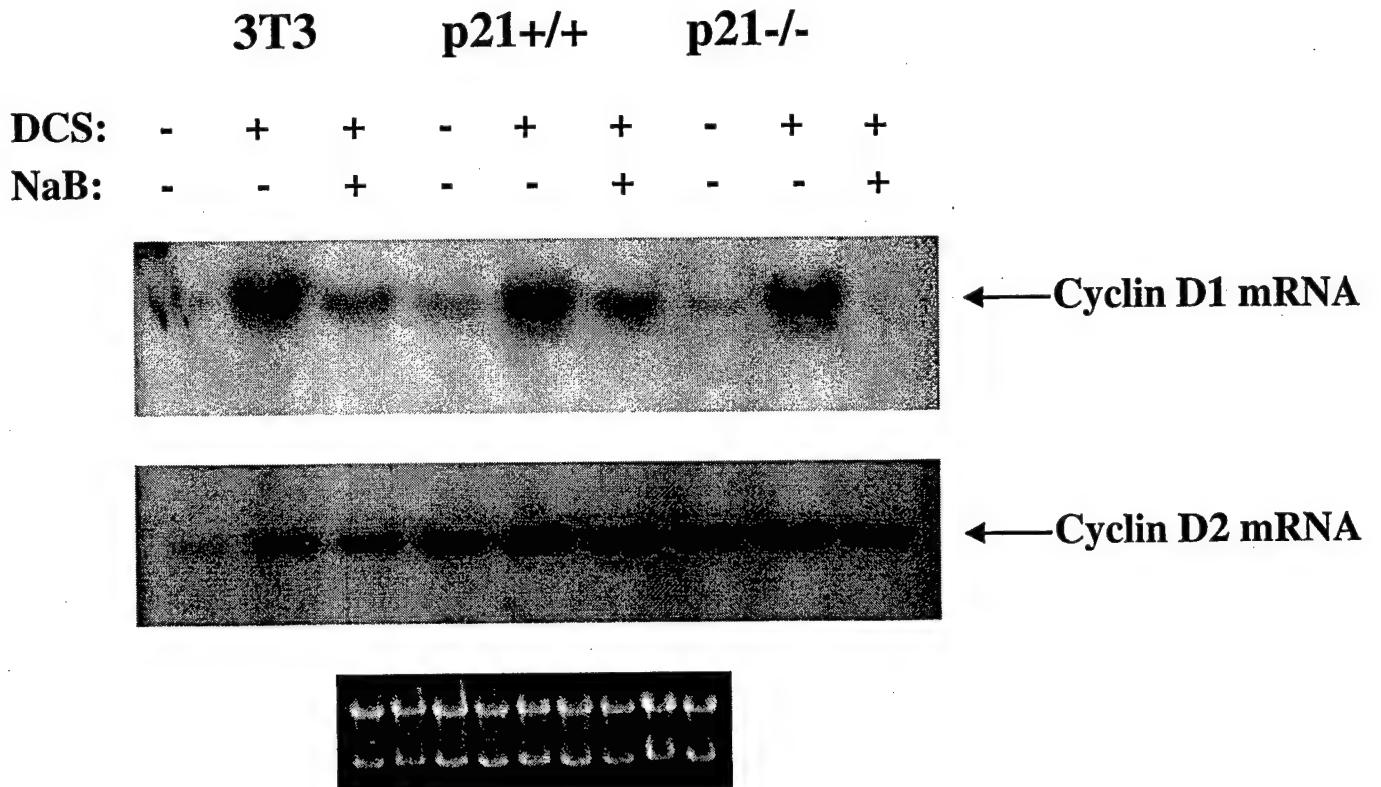


Figure 5. Effect of butyrate on mitogen-induced cyclin D expression in p21+/+ and p21-/- MEFs

(b) Quiescent cultures of p21-/- MEFs were treated without or with 10% serum, in the presence of varying concentrations of sodium butyrate for 8 hours. At this time cytosolic extracts were prepared from the cells. 100 µg of each cytosolic extract was separated on a 10% polyacrylamide gel. After transfer to nitrocellulose, samples were probed with polyclonal antisera to cyclin D₁ as described in 'Materials and Methods'.

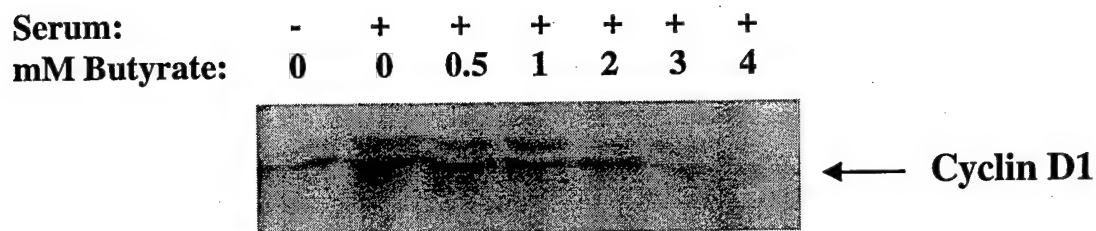


Figure 6. Effect of butyrate on transiently-transfected cyclin D₁ promoter-driven reporter gene activity.

3T3 cells in 10 cm culture dishes were transfected overnight with 10 µg of -1745 D₁-Luciferase and 20 µg of -373 PDGFβR-CAT using co-precipitation of plasmid DNAs with calcium phosphate. Transfected cells were placed in medium containing reduced (0.5%) serum to elicit growth arrest. The resulting quiescent cells were stimulated with 10% serum in the absence or presence of 5mM sodium butyrate for 12 hours. Cytosolic extracts were prepared. A portion of each lysate was assayed for Luciferase (top panel) and CAT (lower panel) activity. The inset panel is an autoradiogram of the developed TLC plate used to separate the products of the CAT reaction. The arrow indicates the position of the di-acetylated [¹⁴C]- chloramphenicol product.

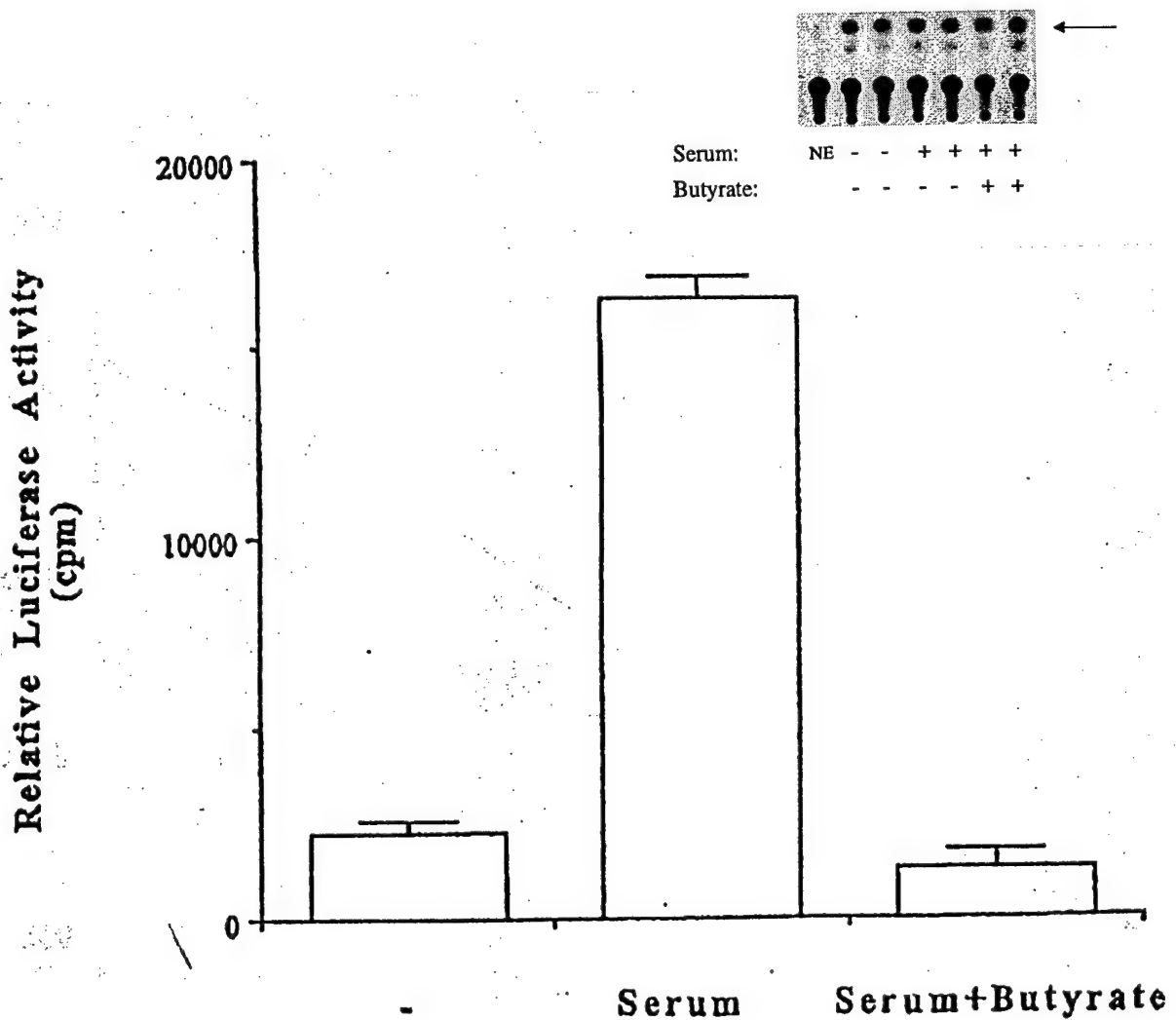


Figure 7. Effects of butyrate on mitogen-dependent Rb phosphorylation and expression of cyclins E and A in 3T3 fibroblasts

(a) Quiescent cultures of p21^{+/+} and p21^{-/-} cells were stimulated with 10% serum without, or with 5 mM sodium butyrate for 13 hours. At this time cytosolic extracts were prepared from the cells. 100 µg of each cytosolic extract was separated on a 6% polyacrylamide gel. After transfer to nitrocellulose, samples were probed with polyclonal antisera to Rb. Bound antibodies were visualized using an ECL kit (Amersham). The arrows indicate the position of the slowly-migrating hyperphosphorylated Rb band.

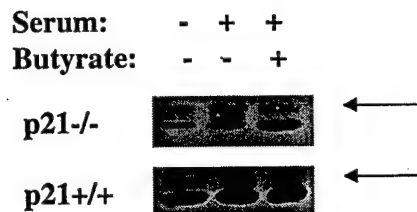


Figure 7. Effects of butyrate on mitogen-dependent Rb phosphorylation and expression of cyclins E and A in 3T3 fibroblasts

(b) A construct containing the promoter region of the murine cyclin E gene upstream of a firefly luciferase cDNA was stably transfected into 3T3 cells as described in 'Materials and Methods'. Quiescent cultures of stably transfected cells were stimulated without or with 10% serum and varying doses of sodium butyrate for 13 hours. Detergent lysates from the cells were normalized for protein content and assayed for luciferase activity as described in 'Materials and Methods'. In the experiment shown, luciferase activity in unstimulated cells (which received neither serum nor butyrate) was 238 ± 146 cpm.

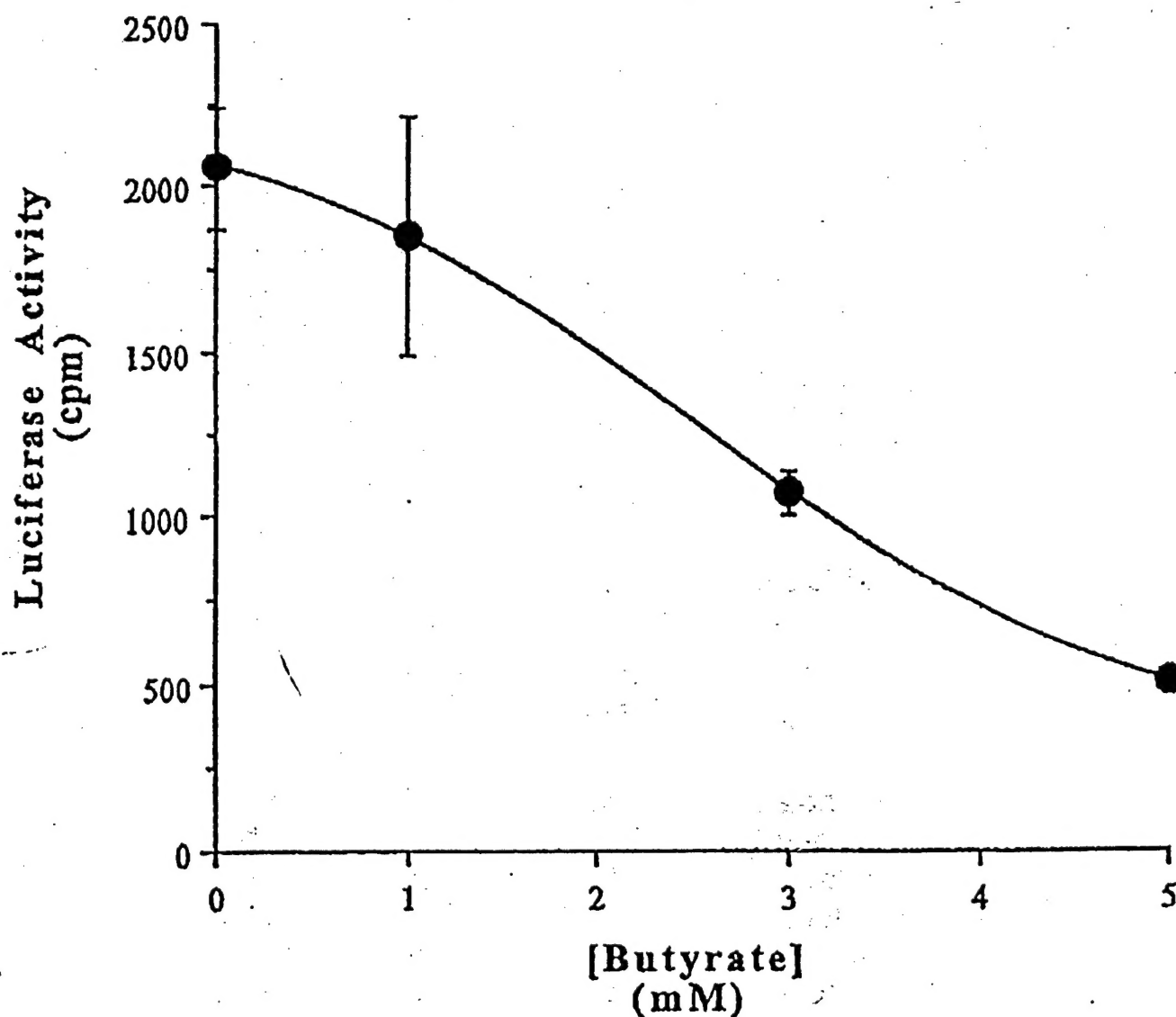


Figure 7. Effects of butyrate on mitogen-dependent Rb phosphorylation and expression of cyclins E and A in 3T3 fibroblasts

(c) Quiescent cultures of 3T3 cells were stimulated with 10% serum without, or with 5 mM sodium butyrate for 13 hours. At this time cytosolic extracts were prepared from the cells. 100 µg of each cytosolic extract was separated on a 6% polyacrylamide gel. After transfer to nitrocellulose, samples were probed with polyclonal antisera to Rb (top panel), cyclin E (middle panel), or cyclin A (lower panel). Bound antibodies were visualized using alkaline phosphatase-conjugated secondary antisera.

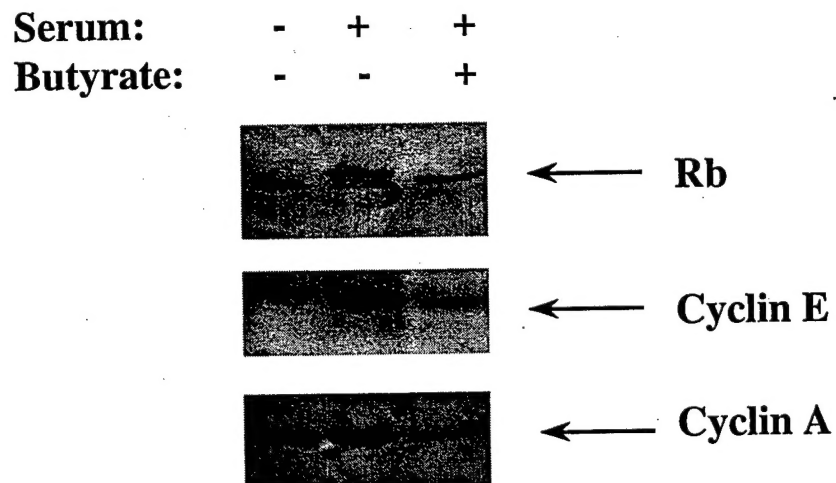


Figure 8. Effect of HPV oncoprotein expression on sensitivity to butyrate-induced G_1 arrest in 3T3 cells

Retroviral vectors were used to express HPV E6, E7, and E6 and E7 cDNAs in 3T3 cells. As a control, 3T3 cells were infected with an 'empty' retroviral vector lacking an ectopic gene. The resulting cells were designated SXS_N (○), SE6 (●), SE7 (△), and SE6E7 (▲), and expressed 'empty' vector, E6 cDNA, E7 cDNA, and E6 plus E7 cDNAs respectively. Cultures of serum-starved cells containing 'empty' vector, or viral oncoproteins, were stimulated with 10% serum for 24 hours without or with varying concentrations of sodium butyrate and 1 μ Ci/ml of [3 H]-thymidine. After 24 hours, the incorporation of tritiated thymidine into genomic DNA was determined by scintillation counting of solubilized nuclei. These values are expressed as % maximal serum-stimulated mitogenic response in the absence of butyrate. Data points are means of duplicate determinations which differed by less than 5%. Error bars have been omitted for clarity.

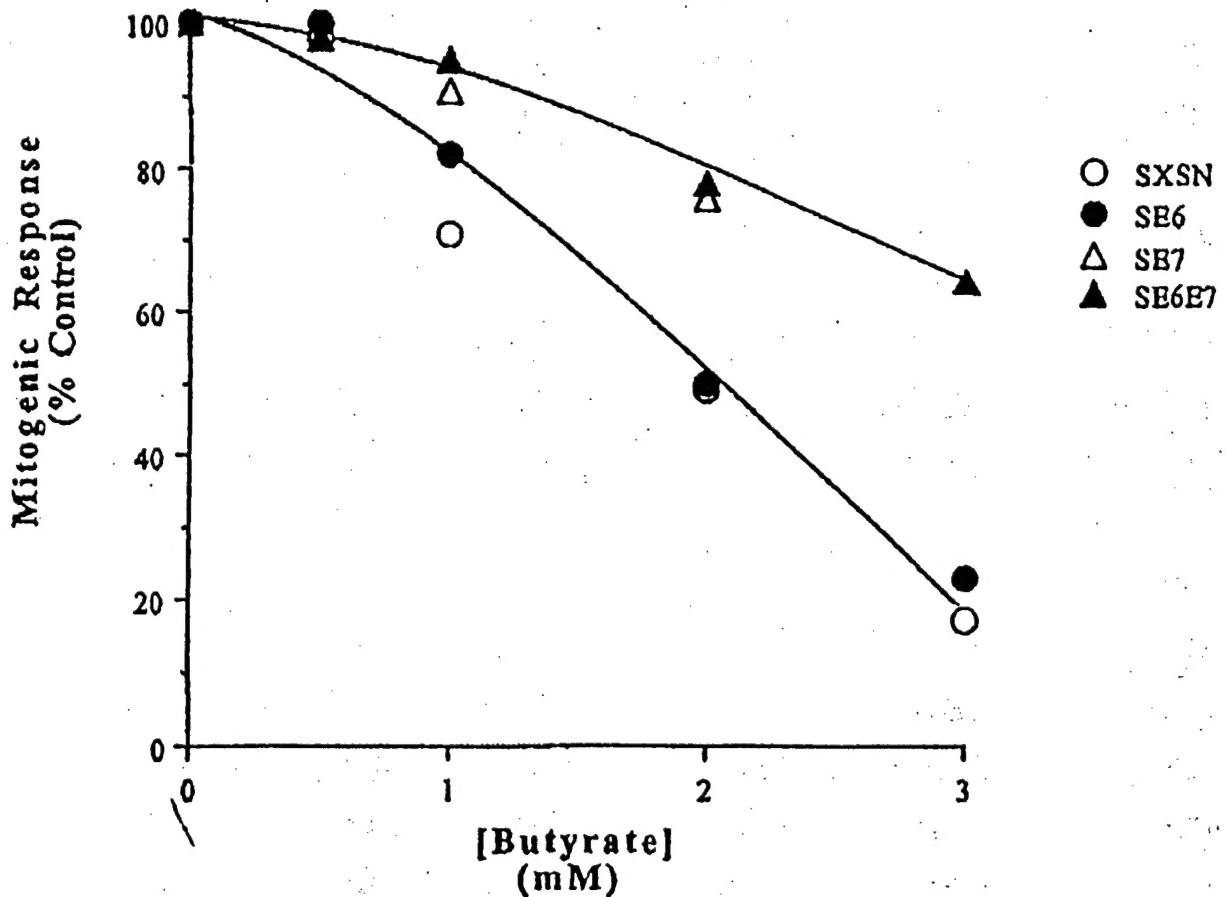


TABLE I

<u>Patient</u>	<u>Age</u>	<u>Site</u>	<u>EBV status</u>	<u>Prior Rx</u>	<u>Hospital</u>	<u>Butyrate+GCV Treatment</u>	<u>Response</u>	<u>Adverse Events</u>
1	48	Thorax	(+)	Cx,XRT,IF	BWH	10 days	Complete	None
2	24	Brain	(+)	Cx,XRT,IF	DFCI	10 days	Complete	None
3	48	Nasal	(+)	Cx,XRT	Paris	21 days	Complete	Supor
4	3	Diffuse	(+)	Cx,XRT,BMT	MSK	7 days	Partial	None
5	30	GI	(+)	Cx,XRT,IF	MSK	21 days	Complete	Lethargy
6	22	Diffuse	(+/-)	Cx,XRT,BMT	Yale-NH	21 days	None	Nausea
7	7	GI	(+)	Gancicovir	Childrens	21 days	Stable Dis.	Nausea

Cx=Chemotherapy

XRT=Radiotherapy

IF=Interferon

BMT=Bone Marrow Transpl.